A System for Characterizing Cellular and Molecular Events in Programmed Neuronal Cell Death

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A model system has been established in which PC12 cells are converted to neuronal-like cells that undergo transcription-dependent cell death following removal of NGF. Nineteen sublines of PC12 cells were tested to establish parameters for making cells dependent on NGF for survival. In most sublines, a relatively small percentage of cells become dependent on NGF for survival, and following removal of NGF, most of the cells begin proliferating in serum-containing medium. In several sublines, however, a significant percentage of cells die following removal of NGF. One of these sublines, PC6-3, can be grown under conditions in which 90% of the cells undergo transcription-dependent cell death following removal of NGF in either serum-free or serum-containing medium. Fourteen hours after removing NGF, 50% of the cells are committed to die, while initial morphological signs of cell death as determined by time-lapse videomicroscopy occur 2-6 hr later and include loss of neurites followed by a 1-3 hr period of active membrane "blebbing" and protrusions. Cell death can be blocked by the RNA synthesis inhibitor actinomycin D, the protein synthesis inhibitor cycloheximide, KCl, basic fibroblast growth factor, or dibutyryl-cAMP, but not by epidermal growth factor, leupeptin, or the endonuclease inhibitor aurintricarboxylic acid (ATA). Removal of NGF activates an endonuclease that causes nucleosomal laddering of the DNA; however, endonuclease activity does not appear to be required for cell death. In agreement with previous studies (Batistatou and Greene, 1991; Rukenstein et al., 1991) demonstrating that naive PC12 cells undergo transcription-independent cell death when shifted into serum-free medium in the absence of growth factors, all cell lines tested except for one die when cultured in RPMI medium lacking growth factors. DNA fragmentation is a prominent feature of transcription-independent cell death, and death can be blocked with NGF, ATA, and dibutyryl-cAMP but not with actinomycin D or KCl. The PC12 model system described here should be useful for identifying cell death genes and for characterizing cellular and molecular events in programmed neuronal cell death.

[Key words: apoptosis, programmed cell death, PC12 cells, differentiation, NGF, RNA synthesis, protein synthesis, transcription dependence]

Cell death in the developing nervous system is extensive and appears to result from a cascade of molecular and cellular events that occur at a specific stage of differentiation (for review, see Clarke, 1990; Ellis et al., 1991; Oppenheim, 1991). In some systems it appears that growth factors such as NGF can block cell death (Martin et al., 1988), while in other systems it appears that cell death is part of a developmental program that is not altered by growth factors (Ellis and Horvitz, 1986). In the vertebrate nervous system approximately half the neurons generated in many neuronal populations die over a relatively short period of time, typically during the period when synapses are being formed between neurons and their targets (Oppenheim, 1991). The emerging view is that developing neurons compete for a limited amount of or limited access to target-derived trophic factors. Trophic factors appear to enhance survival not by supporting life, but rather by blocking an endogenous cell death program (Martin et al., 1988; Oppenheim et al., 1990; Scott and Davies, 1990). Neurons that die during development (and possibly latter in life during aging and in diseases such as Alzheimer's or amyotrophic lateral sclerosis) may do so because they are unable to obtain sufficient quantities of trophic factors to block the endogenous cell death program.

There is considerable interest in identifying neuronal cell death genes. At present, several genes have been identified that appear to be involved in neuronal cell death. In *Caenorhabditis elegans*, 14 genes have been characterized that are involved in various aspects of programmed cell death (Sulston, 1976; Sulston and Horvitz, 1976; Hedgecock et al., 1983; Ellis and Horvitz, 1986; Yuan and Horvitz, 1990; Ellis et al., 1991; Hengartner et al., 1992). Three of these, ced-3, ced-4, and ced-9, are particularly interesting. Mutations in ced-3 or ced-4 genes prevent programmed cell death during development, which suggests that these genes play an active role in determining which cells die. The ced-9 gene product inhibits the function of ced-3 and ced-4 genes, and appears to be required to prevent normal cells from undergoing programmed cell death (Ellis et al., 1991; Hengartner et al., 1992). In *Manduca sexta* polyubiquitin is expressed in neurons and intersegmental muscles that are undergoing programmed cell death induced by a decline in circulating levels of 20-hydroxyecdysone (Schwartz et al., 1990a,b). The role of polyubiquitin in neuronal cell death, however, is not clear.

Little progress has been made in identifying genes involved in neuronal cell death in vertebrates; however, studies by Martin et al. (1988) and Scott and Davies (1990) using primary cultures of neurons and Oppenheim et al. (1990) using motoneurons in the chick lumbar spinal cord *in vivo* suggest that cell death of vertebrate neurons requires new RNA and protein synthesis. None of the genes or proteins involved in cell death of vertebrate neurons have been identified, and thus far the only protein that

has been suggested to be involved in cell death of vertebrate neurons is an endonuclease that is activated when naive PC12 cells are shifted into serum-free medium in the absence of NGF or other growth factors (Batistatou and Greene, 1991; Ruckenstein et al., 1991). The cell death in this case, however, does not appear to require new RNA or protein synthesis; therefore, the relationship between endonuclease activity and "programmed" neuronal cell death is not yet clear. Interestingly, nuclease activity appears to be involved in cell fragmentation during phagocytosis in late stages of programmed cell death in *C. elegans* (Sulston, 1976; Hevelone and Hartman, 1988), and in glucocorticoid-induced cell death of T-lymphocytes (Cohen and Duke, 1984; Wyllie et al., 1984; Shi et al., 1989); therefore, endonuclease activity may be a common feature of many types of cell death. Whether endonuclease activity is the cause or the result of cell death is an area of active research (Cohen and Duke, 1984; McConkey et al., 1989; Schwartzman and Cidlowski, 1991).

The number of genes and proteins involved in neuronal cell death in vertebrates is unknown. As genes are identified it will be increasingly important to understand their role in cell death and their relationship to other genes and proteins that are likely to make up a cascade of events resulting in cell death. *In vitro* model systems are needed for identifying genes and for understanding the various steps in a neuronal cell death cascade. A useful model system would consist of a homogeneous neuronal population that is easily available in large quantities and undergoes transcription-dependent cell death with a precise time course either as part of a developmental program or following trophic factor removal. At present, there are no systems that have all of these properties, although primary cultures of sympathetic neurons meet all the requirements except that large quantities of cells are not readily available. Primary cultures of rat sympathetic neurons require NGF for survival and, following removal of NGF, die 24–48 hr later (Martin et al., 1988). Cell death can be blocked by inhibiting RNA or protein synthesis, suggesting that cell death following removal of NGF requires new RNA and protein synthesis (i.e., a cell death program is activated upon removal of NGF).

The present study started with the hypothesis that if a neuronal cell line could be made dependent on a trophic factor such as NGF for survival, then it might serve as a good model system for identifying the cellular and molecular events responsible for cell death. By definition, cell lines are immortal; therefore, the initial step would be to establish conditions in which a neuroblastoma cell line would become dependent on a trophic factor for survival. Such a system would be useful not only for studying events in neuronal cell death but also for investigating molecular events involved in the irreversible commitment to a postmitotic phenotype. The pheochromocytoma cell line PC12 was chosen because of its well-characterized response to NGF (Greene and Tischler, 1976; for review, see Halegoua et al., 1991; Levi, 1991) and because of its similarity to sympathetic neurons, at present the best culture model of neuronal cell death (Martin et al., 1988; Koike et al., 1989; Wallace and Johnson, 1989; Chang et al., 1990; Martin et al., 1990).

Materials and Methods

Materials. Basic fibroblast growth factor (bFGF) was purchased from GIBCO–Bethesda Research Labs; fibronectin, from Boehringer Mannheim; laminin, from Collaborative Research; Protein A Sepharose, from Pharmacia; and dibutryl-cAMP (dbcAMP), aurintricarboxylic acid

(ATA), insulin, selenium, transferrin, progesterone, putrescine, polylysine, actinomycin D, cycloheximide, leupeptin, and 3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), from Sigma. Culture media were purchased from JRH Scientific (high-glucose DMEM, RPMI, and Ham's F12), GIBCO [minimum essential medium (MEM) and Ham's F12], or Sigma (L15). Horse and fetal calf serum were obtained from HyClone. NGF and epidermal growth factor (EGF) were prepared from male mouse saliva according to the method of Burton et al. (1978), or were purchased from UBI or Collaborative Research. Type I collagen was prepared from rat tails or purchased as bovine skin collagen from Sigma.

Cell culture

Nineteen sublines of PC12 cells were used in these studies. Three of these lines were isolated in 1986 and are designated PC2 (cells grow in small aggregates and have a rapid and robust response to NGF), PC4 (cells grow in amorphous large aggregates and exhibit weak morphological response to NGF; neurites grow within aggregates rather than on substrates), and PC6 (cells grow as single isolated cells, and have a slow but good morphological response to NGF). The other 16 lines were isolated by subcloning the PC6 parent line, and are designated PC6-1 through PC6-16.

PC12 cells were typically maintained in RPMI medium in 10% horse serum and 5% fetal calf serum and grown on tissue culture plastic (Corning). When cells were treated with NGF or used in experiments, they were grown on a matrix (generally type I collagen from rat tail or bovine skin). A large number of different growth conditions and substrates were tested in these studies; information on some of these different conditions are given in Results. The protocol presently being used to convert PC12 cells to a dependence on NGF for survival is as follows: the PC6-3 subline of PC12 cells are grown on type I collagen in RPMI medium containing 5% fetal calf serum and 10% horse serum for 7–10 d in the presence of 100 ng/ml 2.5S NGF with fresh medium being added every 2 d. On day 7 or 10 the cells are removed from the dishes with Hank's balanced salt solution and trituration and replated onto dishes containing type I collagen in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) serum-containing medium (5% or 10% fetal calf serum) containing 100 ng/ml 2.5S NGF, or serum-free medium (DMEM/F12) containing insulin (5 μg/ml), transferrin (10 μg/ml), selenium (30 μM), progesterone (20 nM), putrescine (100 μM), BSA (100 μg/ml), and 2.5S NGF (100 ng/ml). After an additional 7 d, NGF is removed from cultures by adding medium lacking NGF (serum-free or serum-containing depending on the culture conditions during the last 7 d) and containing 60 μg/ml anti-NGF IgG. Because NGF is a "sticky" molecule, the addition of antibodies provides a more precise time point for removal of the biological effects of NGF. The antibodies only "tighten up" the time course of cell death (without antibodies cells die between 24 and 96 hr following removal of NGF; see Fig. 1; see also Martin et al., 1988).

Assays for cell survival

Lactate dehydrogenase. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was used as a measure of cell viability for some cultures grown in serum-free medium (serum contains LDH, which interferes with the assay). LDH activity was determined using a colorimetric assay (LDH diagnostic kit, Sigma) based on the conversion of pyruvic acid to lactic acid by LDH in the presence of NADH (Cabaud and Wroblewski, 1958). The assay was performed according to the manufacture's protocol. Total cellular LDH was determined by freezing and thawing cells two times.

MTT. A modification (Hansen et al., 1989) of the original procedure (Mosmann, 1983) using the tetrazolium salt MTT [3,(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide] to measure mitochondrial function was used. In short, MTT (1 mg/ml final concentration) was added to cultures of PC12 cells growing in serum-free or serum-containing medium in 24-well dishes (10,000–30,000 cells/well) and incubated at 37°C for 1 hr. The assay was stopped by lysing cells with 20% SDS in 50% N,N-dimethyl formamide, pH 4.7. This was then incubated overnight at 37°C, transferred to a microtiter plate, and quantitated using an ELISA plate reader at 570 nm. The assay was linear between 1000 and 40,000 cells and 45 and 150 min reaction time and was very reproducible between experiments.

Cell counts. Cells grown in 24-well culture dishes were counted at 200× magnification. Cell counts for each experimental condition consisted of five fields/well (center and 12, 3, 6, and 9 o'clock) and two or
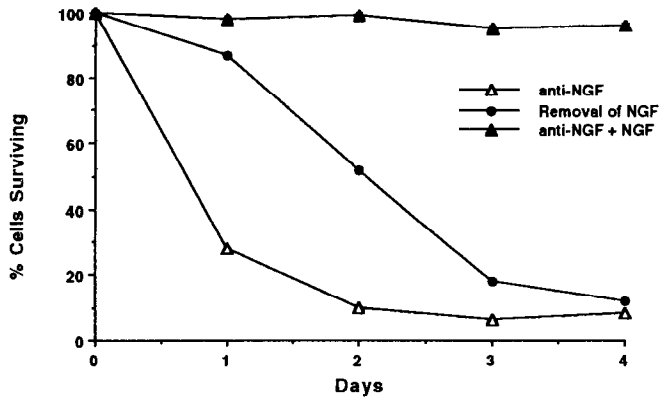
Figure 1. Cell death following removal of NGF in the absence (*solid circle*) or presence (*open triangles*) of antibodies directed against NGF. Data represent the means of cell counts (relative to the number of cells present in controls at the start of the experiment) of 10 fields for two cultures in two different platings. In the anti-NGF + NGF group (*solid triangles*), NGF was removed and 60 μg/ml anti-NGF added, followed 4 hr later by the addition of 400 ng/ml NGF.

Table 1. Cell death of PC6 sublines following removal of NGF

Subline	% Survival	Protection with actinomycin D	Background cell death
1	23	***	Medium
2	91	—	Low
3	4	***	Low
4	29	**	Medium
5	44	***	Low
6	30	**	Low
7	38	**	Low
8	21	**	High
9	7	*	High
10	19	***	High
11	24	***	Medium
12	32	**	High
13	19	**	High
14	34	**	Low
15	8	***	Medium
16	37	*	High

The percentage survival was determined for 10 fields of cells in two cultures for each subline. Cell counts were made 36 hrs after removal of NGF. Background cell death was determined prior to removal of NGF and considered low if 0–3% of the cells/field were degenerating, medium if 4–7% of cells were degenerating, and high if 8–15% of the cells were degenerating. Addition of actinomycin D at the time of NGF removal provided essentially complete protection (***), partial protection (**), or very little protection (*) after 36 hr.

three wells/experiment. Therefore, 10–15 fields containing 10–75 cells each constituted $N = 1$. Living cells were determined by phase-contrast characteristics of a smooth phase-bright cell body with clear cytoplasm. Strong correlations were obtained when comparing cell survival obtained from cell counts with survival based on the lactate dehydrogenase assay ($r = 0.97$) or MTT assay ($r = 0.94$).

DNA laddering

Soluble DNA was prepared from PC12 cells by the method of Davis et al. (1980). Approximately 5×10^6 cells were solubilized at 60°C for 2 hr in isotonic saline containing 0.1 M EDTA, 1% SDS, 100 μg/ml Proteinase K, and 0.2 M Tris, pH 8.5, followed by precipitation of chromosomal DNA with 5 M potassium acetate and centrifugation for 15 min at 15,000 × g. Soluble DNA was precipitated from the supernatant with ice-cold ethanol and resuspended in 10 mM Tris/1 mM EDTA, pH 8.0, and the concentration of DNA was determined by UV absorbance at 260 nm. Equal amounts of DNA were run in each lane of a 2% agarose gel.

Time-lapse videomicroscopy

Cells were cultured on collagen-coated glass coverslips attached to the bottom of 35 mm dishes in which a 1 cm hole had been drilled (MatTek Corp., Ashland, MA). Cultures were grown on the stage of a Nikon Diaphot microscope under 5% CO_2 by passing a mixture of 5% CO_2, 95% air through a port on the side of a specially designed culture chamber and maintained at 37°C by having the entire apparatus in a temperature-controlled warm room. Videomicroscopy was performed using a Panasonic videocamera with newvicon tube and Panasonic AG-6050 time-lapse video recorder. Images were enhanced using an Image 1/AT digital image processing system (Ivins and Pittman, 1989; Ivins et al., 1991).

Antisera

Antisera against NGF were prepared by injecting New Zealand White rabbits with 200 μg of 2.5S NGF in Freund's complete adjuvant, followed by two boosts 6 weeks apart with 100 μg of 2.5S NGF in Freund's incomplete adjuvant. Serum was collected from the marginal ear vein every 4 weeks and an IgG fraction prepared using Protein A Sepharose.

Results

Characterizing sublines and culture conditions

Initial studies indicated that 10–20% of two sublines, PC2 and PC6, became dependent on NGF for survival after 14 d of culturing on "native" substrates of laminin, fibronectin, type I collagen, and type IV collagen, but not on polylysine or tissue

culture plastic. One subline, PC4, did not become dependent on NGF under any of the conditions tested. Several media (RPMI, F12, DMEM, L15, MEM, and DMEM/F12 mix) and growth factors (bFGF, EGF, and dbcAMP) were tested using PC2 and PC6 sublines cultured under a variety of experimental paradigms. From these experiments it was determined that for maximum dependence on NGF for survival, cells should be grown on type I collagen in a medium designed for PC12 cells consisting of RPMI medium containing 10% horse serum, 5% fetal calf serum, and 100 ng/ml NGF for the first 7–10 d, and then subcultured onto type I collagen and grown in a medium designed to maintain primary cultures of neurons consisting of a 1:1 mixture of DMEM/F12 containing 5% or 10% fetal calf serum, or in serum-free DMEM/F12 containing N2 supplements (Bottenstein et al., 1980). The PC6 subline was chosen for further studies because of the lower spontaneous background cell death associated with these cells compared to the PC2 subline. Because spontaneous background cell death would likely complicate future biochemical and molecular studies, cell lines and conditions were chosen to minimize background death.

Under the paradigm described above, approximately 50% of the PC6 cells became dependent on NGF for survival. In an attempt to increase the percentage of cells dying following removal of NGF, PC6 cells were subcloned into 16 stable sublines and examined for their dependence on NGF for survival, for the ability of actinomycin D to block cell death, and for spontaneous background cell death (Table 1). One of these lines, PC6-3, had low spontaneous background cell death, greater than 90% of the cells became dependent on NGF for survival, and most of the cell death could be blocked with actinomycin D.

Transcription-dependent cell death in PC6-3 cells

Removal of NGF from cultures of PC6-3 cells results in about 90% of the cells dying over a period of 3–4 d (Fig. 1). Because
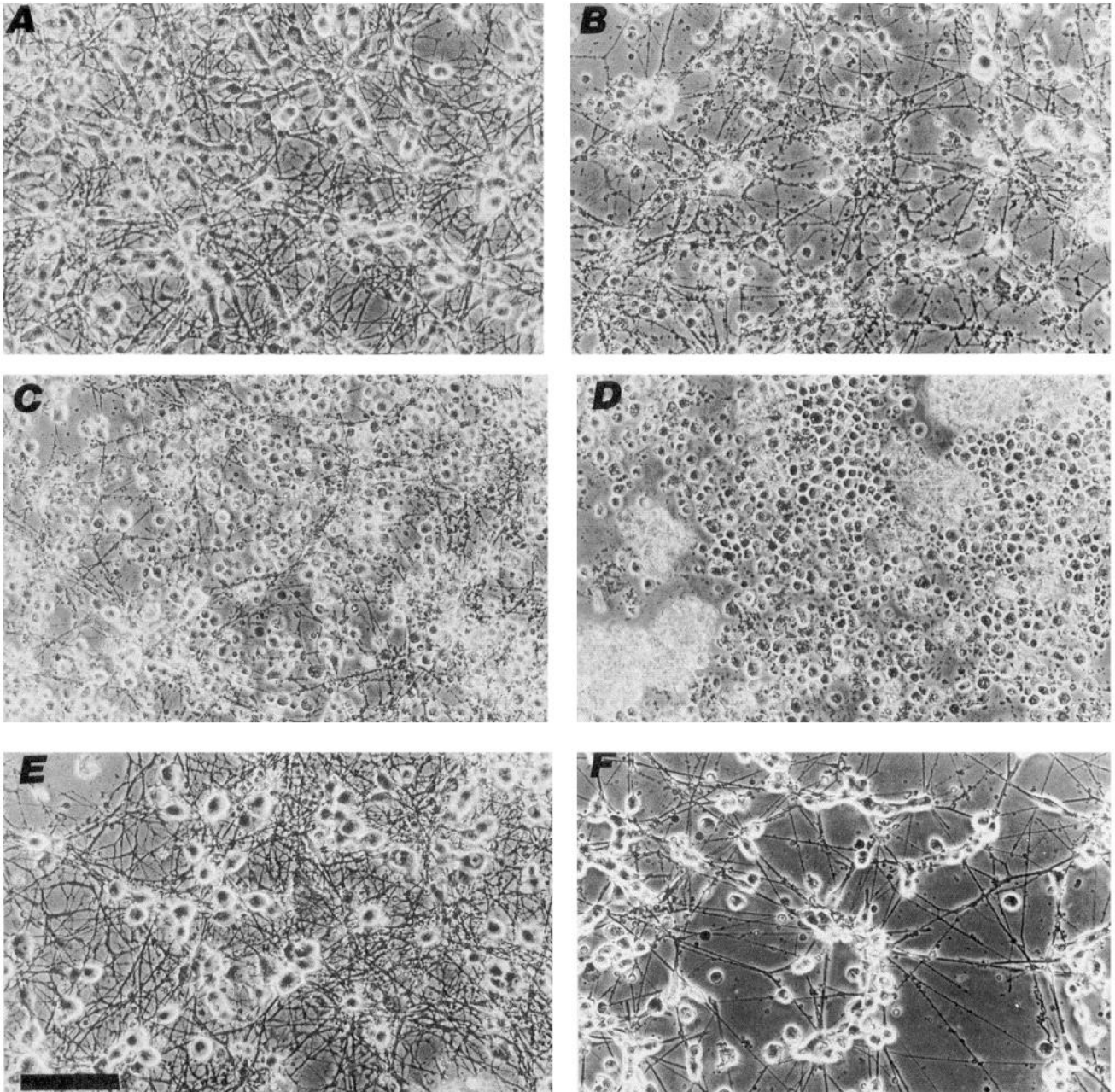
Figure 2. Cell death of PC6-3 cells following removal of NGF and protection with actinomycin D: control cells in the presence of NGF (*A*), or 20 hr (*B*), 30 hr (*C*), or 72 hr (*D*) following removal of NGF. Actinomycin D (2 μg/ml) added at the time of NGF removal protects cells from death for 30 hr (*E*) and 72 hr (*F*). Cells were grown in serum-free medium during the last 7 d of culturing; similar data were obtained with cells grown in serum-containing medium. Scale bar, 100 μm.

NGF is a "sticky" molecule, the protracted period over which cells die may be due to the continued presence of NGF bound to serum proteins, cells, or the culture dish (see also Martin et al., 1988). When 60 μg/ml anti-NGF IgG is added to cultures, the time course of cell death is shortened considerably (Fig. 1). The possibility that antibodies bind to surface-bound NGF and are internalized and produce nonspecific effects was tested by exposing cells to antibodies for 4 hr (sufficient time for internalization of surface-bound NGF and antibodies) followed by adding a fourfold excess of NGF to the cultures. No cell death occurs during the 4 d period when cells are treated with antibodies followed by excess NGF (Fig. 1). As an additional control to rule out nonspecific effects of antibodies, antibodies directed

against tissue plasminogen activator (tPA; a soluble molecule present in the culture that binds to the surface of PC12 cells; Pittman et al., 1989) were added to cultures of differentiated PC12 cells. Antibodies against tPA did not alter the time course of cell death following removal of NGF (R. N. Pittman, S. Wang, A. DiBenedetto, and J. Mills, unpublished observations). In all subsequent experiments, anti-NGF IgG is included in medium when NGF-containing medium is removed.

After 14 d in the presence of NGF, PC6-3 cells appear very neuronal (Fig. 2A). Following removal of NGF, the first morphological signs of cell death at the light microscopic level occur after about 16 hr and involve the thinning and "beading" of some of the neurites. By 20 hr after removal of NGF, a number
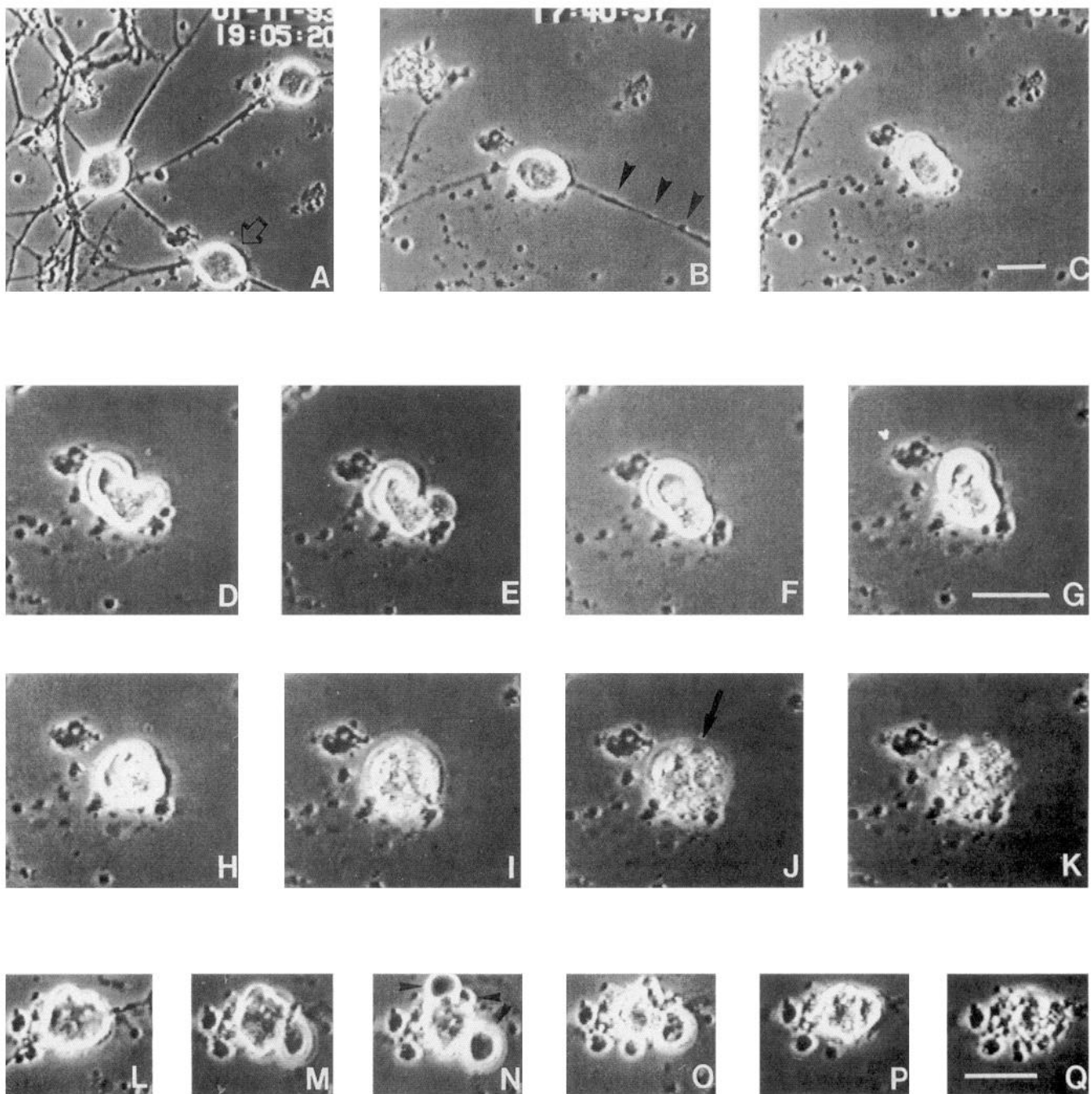
Figure 3. Morphological changes occurring after removing NGF. Continuous time-lapse videomicroscopy was performed on cell cultures following removal of NGF. The cell in the lower part of *A* (*open arrow*) is depicted at various stages of cell death (*A–K*). Thinning and "beading" of neurites (*arrowheads, B*) begins 26 hr after removing NGF and neurites have essentially disappeared 35 min later (*C*; a very thin neurite ~0.1 μm in diameter is all that remains). Constant changes in the shape of the cell body occur over the next 2.5 hr (*C–G*, taken at 30 min intervals). This is followed by a period of 3–4 hr of little change in the shape of the cell body (*H*). The cell body then swells slightly (*I*) and appears to burst (*J*, taken 4 sec after *I*; *arrow* points to rupture in plasma membrane), and finally cell debris is all that remains (*K*, taken 16 sec after *J*). *L–Q* depict a slightly different morphology associated with cell death in these cultures. *L* is taken just before neurites degenerate; the cell body has already started to lose its typical smooth, rounded shape. Active protrusion and retraction of "apoptotic-like" bodies occurs following loss of neurites (*arrowheads* in *N* point to some of these protrusions). *M* and *N* are taken 10 and 25 min, respectively, after loss of neurites, and *O*, 3 hr after loss of neurites. After this period of active protrusions, the cell body becomes fairly quiescent for 2–3 hr (*P*), and then swells slightly and ruptures, leaving behind cellular debris (*Q*). Three different magnifications are present: *A–C* (20 μm bar in *C*), *D–K* (20 μm bar in *G*), and *L–Q* (20 μm bar in *Q*).

of neurites have been lost and some of the cell bodies are beginning to degenerate (Fig. 2*B*). By 24–30 hr, extensive loss of neurites occurs and cellular degeneration is obvious (Fig. 2*C*). Over the next 12 hr most of the remaining cells die. The RNA synthesis inhibitor, actinomycin D, blocks the majority of the

cell death when added to cultures at the time of NGF removal (Fig. 2*E*). Even 72 hr after removal of NGF when most cells are dead and much of the cellular debris has detached from the substrate (Fig. 2*D*), actinomycin D still effectively blocks much of the cell death (Fig 2*F*). After 3.5–4 d of treatment, actino-
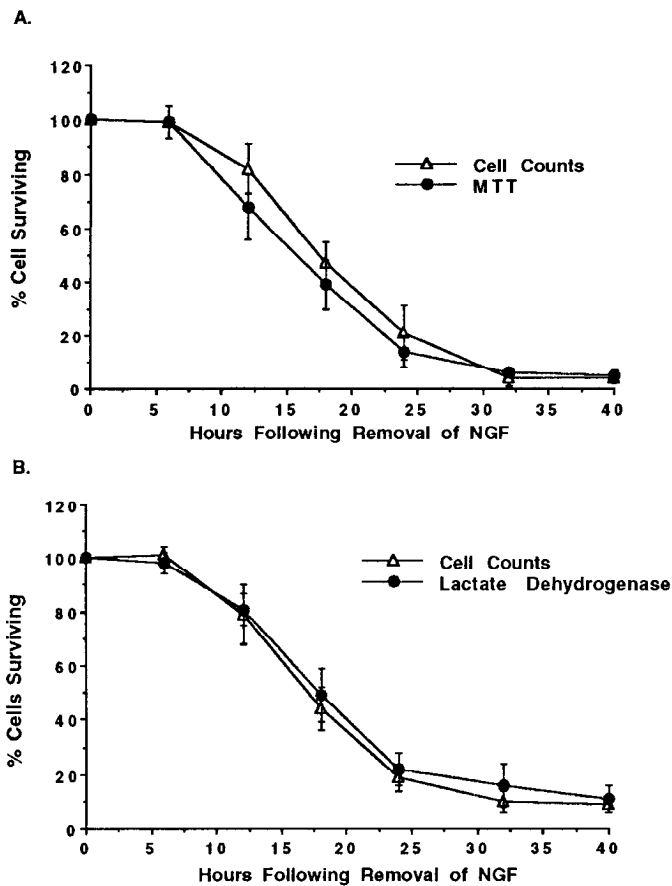
A.

B.

Figure 4. Comparison of different methods for determining cell survival. Data in *A* were taken from cultures grown in serum-containing medium (similar data were obtained for MTT and cell counts in serum-free medium). Data in *B* were taken from cultures grown in serum-free medium for the last 7 d of culturing (serum contains LDH; therefore, LDH activity has been determined only in cultures grown in serum-free medium). Data represent means (±SD) of three different experiments performed in duplicate for cell counts (*open triangles*), MTT (*solid circles* in *A*), and LDH (*solid circles* in *B*). Correlations: cell counts versus MTT, *r* = 0.94; cell counts versus LDH, *r* = 0.97; MTT versus LDH, *r* = 0.92.

mycin D itself is toxic and eventually kills all cells during the next 24 hr in the presence or absence of NGF.

Time-lapse videomicroscopy was performed to examine in greater detail morphological changes occurring after removing NGF. Neurites of the cell indicated by the open arrow in Figure 3*A* begin "beading" (Fig. 3*B*, arrowheads) and thinning 26 hr after removing NGF. About 30 min later, neurites have thinned to fine threads, and the cell body is beginning to alter its shape drastically (Fig. 3*C*). By 27 hr after NGF removal, the cell has lost its neurites and is in the midst of convulsive expansions and contractions (Fig. 3*D–G* taken at approximately 30 min intervals). After 2.5 hr of these dramatic shape changes, the cell returns to a relative quiescent state for about 3.5 hr (Fig. 3*H* is representative of this stage). Then suddenly, over a period of minutes, it begins to swell slightly (Fig. 3*I*) until the plasma membrane appears to lyse (Fig. 3*J*, arrow), and the cell ceases all activity and remains a collection of debris and membrane fragments (Fig. 3*K*). A slightly different form of death typical in these cultures is exemplified in Figure 3*L–Q*. The cell body as a whole seems more stable, but large membrane "blebs" reminiscent of apoptotic bodies seen in various cell types (Clarke,
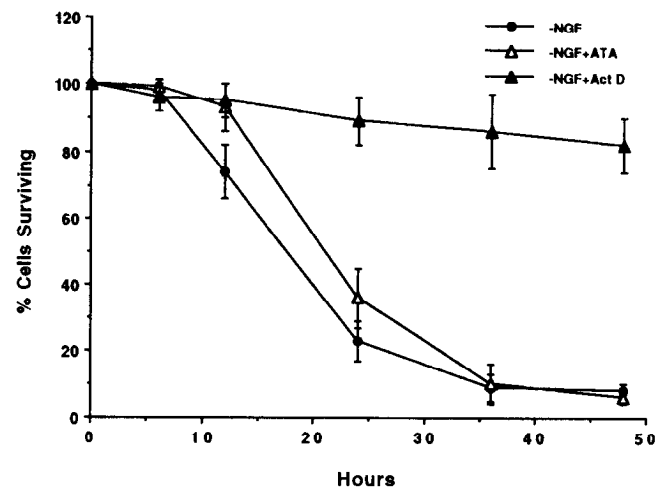

Figure 5. Effect of actinomycin D (2 µg/ml; *solid triangles*) and ATA (30 µM; *open triangles*) on cell death following removal of NGF (*solid circles* reflect survival in the absence of additives). Data represent means (±SD) of three different experiments performed in duplicate and expressed relative to the number of cells present at the start of the experiment. NGF was removed from all cultures at time 0, and cell survival was determined using MTT.

1990) are quickly extended and retracted at multiple sites around the periphery of the cell body (Fig. 3*M–O*; arrowheads in Fig. 3*N*). After a period of 3 hr of many such cycles of "bleb" extension and retraction, the cell exhibits the same morphological changes described above: a quiescent phase (Fig. 3*P*), a swelling (not shown), and finally fragmentation and cell debris (Fig. 3*Q*).

Three different assays have been used to quantitate cell viability (Fig. 4*A,B*). Similar cell survival data is obtained using cell counts, release of the cytoplasmic enzyme LDH, or the tetrazolium dye MTT, which provides a measure of mitochondrial function. Release of LDH is used to quantitate cell survival in serum-free cultures only because of its high background activity in serum-containing cultures. Strong correlations exist when comparing these measures of cell viability (*r* = 0.94, cell counts vs MTT; *r* = 0.97, cell counts vs LDH; *r* = 0.92, MTT vs LDH).

Prevention of cell death following removal of NGF

A requirement for new RNA synthesis has been suggested and/or shown to be required for cell death in a number of systems (Tata, 1966; Lockshin, 1969; Horvitz et al., 1982; Cohen and Duke; 1984; Wyllie et al., 1984; Martin et al., 1988; Oppenheim et al., 1990), and a calcium-dependent endonuclease is activated in a variety of dying cells (Cohen and Duke, 1984; Wyllie et al., 1984; McConkey et al., 1989; Batistatou and Greene, 1991). Cell death of PC6-3 cells following removal of NGF is effectively blocked for at least 48 hr in the presence of the RNA synthesis inhibitor actinomycin D but not in the presence of the endonuclease inhibitor ATA (Fig. 5). These observations are consistent with the idea that cell death in this system requires new RNA synthesis but does not require endonuclease activity. DNA "laddering" into multiples of 180–200 base pair nucleosomes as a result of endonuclease activity is a cardinal feature of apoptotic cell death and is suggested to be the reason for cells dying (McConkey et al., 1989). Although DNA laddering is present 14 hr after removal of NGF from PC6-3 cells (Fig. 6, lane 4), it also occurs to some extent in cells treated with actinomycin D (Fig. 6, lane 5) that do not die during the first 48 hr following
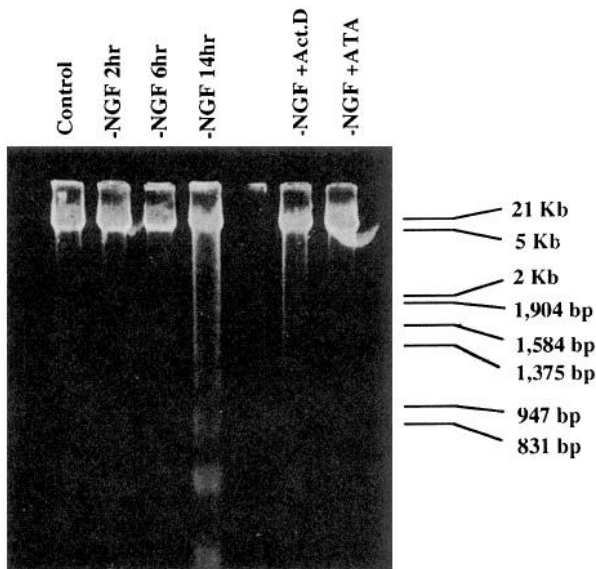
Figure 6. DNA laddering following removal of NGF. A small amount of background DNA laddering is present in control cultures (*lane 1*) and appears to be due to background cell death in these cultures. Following removal of NGF, no increase in DNA laddering occurs during the first 6 hr (*lanes 2, 3*), but is obvious at 14 hr (*lane 4*). Some DNA laddering occurs in the presence of actinomycin D (*lane 5*) but is blocked by ATA (*lane 6*).

removal of NGF (see Fig. 5). In addition, DNA laddering is effectively blocked by ATA (Fig. 6, lane 6), which does not block cell death (see Fig. 5). Therefore, although DNA laddering occurs in differentiated PC6-3 cells following removal of NGF, it does not appear to be required for death of these cells.

Besides actinomycin D, other treatments that block cell death include 35 mM KCl, 1 mM dbcAMP, 20 ng/ml bFGF, and 10 μg/ml cycloheximide (Fig. 7). Agents that have no effect on cell survival include 30 or 100 μM ATA, 10 or 100 ng/ml EGF, and 100 μg/ml leupeptin (Fig. 7). Although leupeptin has no effect on cell survival, cell death in the presence of leupeptin differs from other treatments. Typically, the first morphological changes that occur following removal of NGF are the beading of neurites followed by fragmentation of neurites and death of the cell body. Many cells treated with leupeptin maintain morphologically healthy neurites even though the cell body is compacted and fragmented (most obvious 18–30 hr after removal of NGF; data not shown). By 48 hr after removal of NGF, neurites are fragmented even in the presence of leupeptin, but this typically occurs following demise of the cell body and not prior to changes in the cell body as is more typical of controls and other treatments. The late degeneration of neurites in the presence of leupeptin is probably due to the ability of leupeptin to inhibit calcium-dependent cysteine proteinases (such as the calpains) that are involved in degradation of neurofilaments and other cytoskeletal proteins (Melloni and Pontremoli, 1989).

To define more clearly the time after removal of NGF that cells are irreversibly committed to die, NGF was removed from cells and actinomycin D or NGF added to cultures at 6 hr intervals (Fig. 8) and then the number of living cells was determined after 48 hr. Cells do not exhibit morphological signs of cell death at the light microscopic level 12 hr after removal of NGF; however, 40–50% of the cells are already committed to die at this point. By 18–24 hr, essentially all cells that will
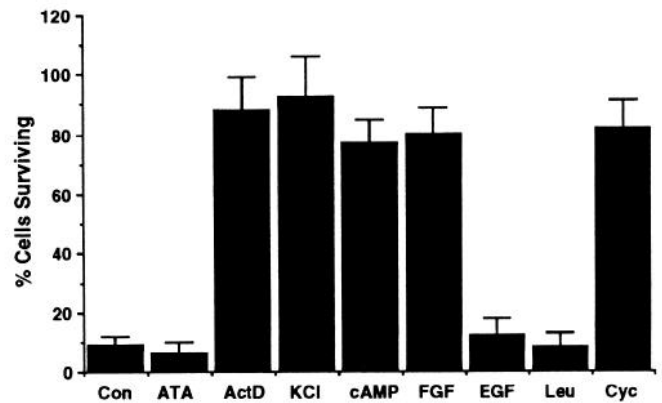

Figure 7. Ability of agents to block cell death following removal of NGF. Data were taken 36 hr after removal of NGF and represent means ($\pm$SD) relative to survival in the presence of NGF of three different experiments performed in duplicate. Agents were added at time 0: 30 μM ATA, 2 μg/ml actinomycin D, 35 mM KCl, 1 mM dbcAMP, 20 ng/ml bFGF, 100 ng/ml EGF, 100 μg/ml leupeptin (*leu*), and 10 μg/ml cycloheximide (*cyc*).

die within the first 48 hr after removal of NGF are committed to die (Fig. 8). These data suggest that 12–14 hr after removal of NGF, half the cells have generated sufficient "killer" mRNA and/or protein to die over the next several hours. The difference in survival between actinomycin D and NGF at early time points is due to a small amount (about 10%) of transcription-independent cell death. Addition of NGF at early times can block transcription-independent death, but addition of actinomycin D cannot. Therefore, no difference was observed for the time of commitment to cell death as defined by NGF or actinomycin D (but see Edwards et al., 1991).

Transcription-independent cell death of PC6 cells

PC12 cells undergo transcription-independent cell death when shifted into serum-free medium lacking growth factors (Batistatou and Greene, 1991; Ruckenstein, 1991). To gain a better understanding of the similarities between the transcription-dependent and -independent cell death of PC12 cells, 16 sublines
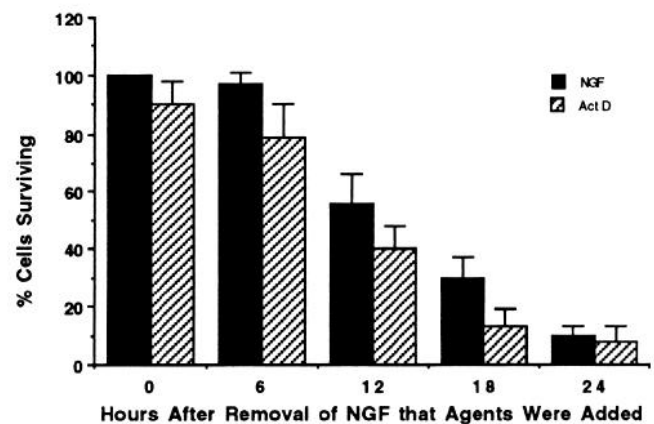

Figure 8. Time of commitment to cell death following removal of NGF. Data are expressed relative to the number of cells in the presence of NGF at time 0 and represent averages ($\pm$SD) of three different experiments performed in duplicate. NGF (100 ng/ml, *solid bars*) or actinomycin D (2 μg/ml, *hatched bars*) were added 0, 6, 12, 18, or 24 hr after removal of NGF from cultures. Cell survival was determined 48 hr after removal of NGF using MTT.
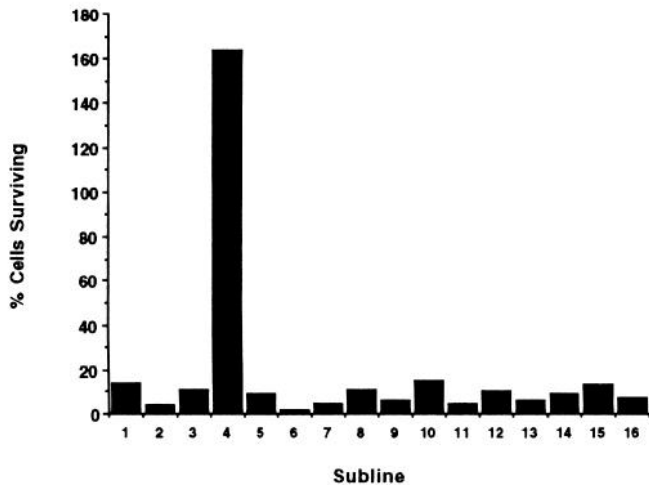
Figure 9. Survival of different sublines of PC6 cells in medium lacking serum and growth factors. Cells were shifted into RPMI medium lacking any additives and cell counts made 5 d later. Data represent the average of two experiments performed in triplicate and are expressed as a percentage of the cells plated.

of PC6 cells (see Table 1) were tested in the transcription-independent cell death paradigm established by Ruckenstein et al. (1991). The majority of cells in most sublines die during the first 4 d in serum-free RPMI medium (Figs. 9, 10A,B). Cells of the PC6-4 subline, however, show very little death in this paradigm (Figs. 9, 10D). This is markedly different from the extensive cell death of PC6-4 cells that occurs following removal of NGF in the transcription-dependent paradigm described above (Table 1).

Transcription-independent cell death of PC12 cells can be blocked by the endonuclease inhibitor ATA (Batistatou and Greene, 1991). PC6-3, PC6-4, PC6-11, and PC6-15 cells were chosen to characterize further the role of endonucleases in transcription-independent cell death. ATA blocks death of PC6-3 cells (Fig. 10C) as well as PC6-11 and PC6-15 cells (data not shown). PC6-3, PC6-11, and PC6-15 sublines show a similar concentration-dependent rescue by ATA, with 30 μM being the most effective concentration for blocking cell death (Pittman, Wang, DiBenedetto, and Mills, unpublished observations). ATA maintains all three sublines for at least 8 d in serum-free RPMI (data for PC6-3, Fig. 11A). The number of PC6-4 cells is greater in the presence of ATA (Fig. 11B), suggesting that some cell death may occur in PC6-4 cells that is blocked by ATA. A small number of degenerating cells are seen in cultures of PC6-4 cells (Fig. 10D), which is consistent with a greatly reduced amount of cell death in these cells rather than a total absence of transcription-independent cell death. This is also supported by the very low but detectable level of DNA "laddering" in PC6-4 cells that is blocked with ATA (Fig. 12, lanes 1–4). DNA laddering occurs in PC6-3, PC6-11, and PC6-15 cells as early as 6–10 hr after cells are shifted into serum-free medium, and extensive laddering is present after 18–24 hr in serum-free medium (Fig. 12, lanes 6, 8). Essentially all of the DNA laddering is blocked with 30 μM ATA (Fig. 12, lanes 5, 7).

Whereas ATA blocks cell death of PC6 cells shifted into serum-free medium, actinomycin D has little or no effect on the rate of cell death (Fig. 13). The inability of actinomycin D to block cell death does not appear to be due to direct toxicity (at least during the first 2 d), since actinomycin D does not significantly reduce the survival enhancing effects of NGF until days 3–5 (Fig. 13). In addition to ATA and NGF, dbcAMP blocks cell death of PC6 cells shifted into serum-free medium (Fig. 14). KCl does not block the death of cells, and significantly reduces survival of PC6-4 cells compared to controls, NGF-treated cells, or dbcAMP-treated cells (Fig. 14).
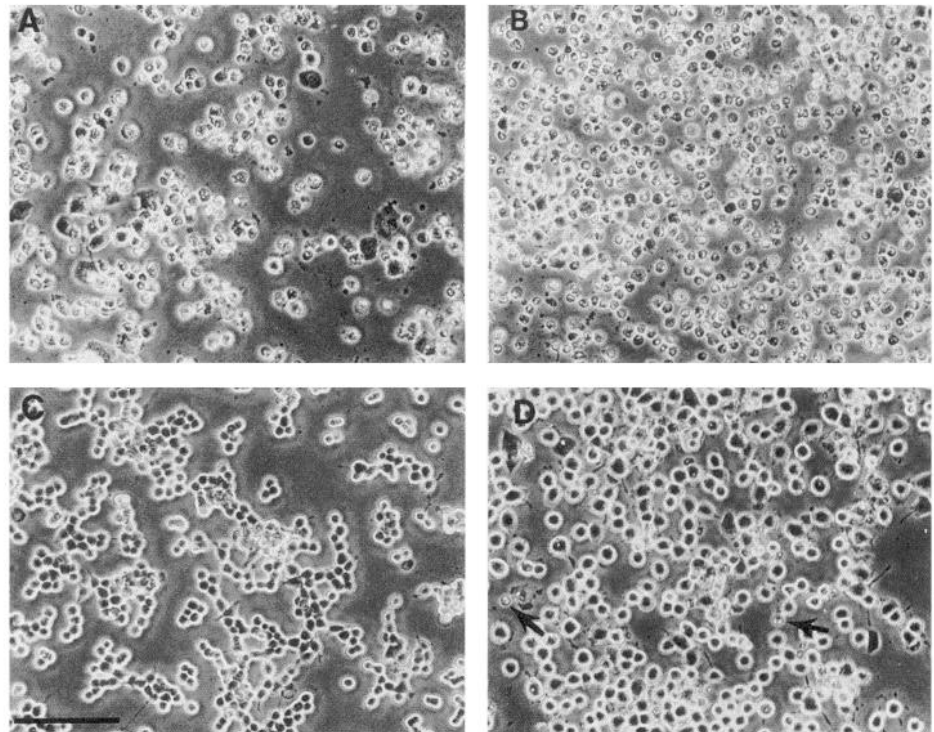
Figure 10. Death of cells in medium lacking serum and growth factors and protection by ATA. *A* and *B*, PC6-11 (*A*) and PC6-3 (*B*) cells in serum-free medium lacking growth factors for 4 d. Note that most cells have degenerated. *C* and *D*, PC6-3 cells after 8 d in medium containing 30 μM ATA (*C*), and PC6-4 cells after 8 d in medium lacking any additives (*D*). Few if any PC6-3 cells die in the presence of ATA, and very few cells in the PC6-4 subline die. *Arrows* in *D* point to a small number of degenerating cells in the PC6-4 subline. Scale bar: 100 μm for *A, B,* and *D*; 200 μm for *C*.
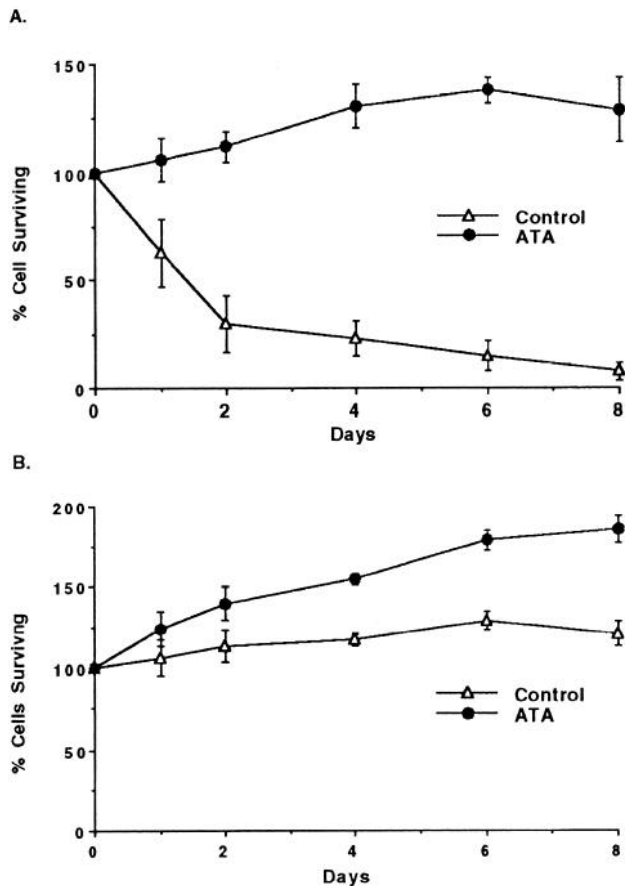
A.

B.

Figure 11. Time course of survival of PC6-3 (*A*) and PC6-4 cells (*B*) in the absence (*open triangles*) or presence (*solid circles*) of ATA in serum-free medium lacking growth factors. Data represent averages (±SD) of three different experiments performed in duplicate and are expressed as a percentage of the number of cells plated. Cell survival was determined using cell counts (two experiments) or MTT (one experiment). Data similar to that for PC6-3 cells were obtained with PC6-11 and PC6-15 cells.

Figure 12. DNA laddering of PC6-4 and PC6-11 cells in the absence and presence of ATA. A large amount of DNA laddering is present in PC6-11 cells 18 and 24 hr after shifting cells into serum-free medium lacking growth factors. ATA is able to block most of the DNA laddering. A small but detectable amount of DNA laddering occurs in PC6-4 cells, and this is blocked by ATA. Similar DNA laddering patterns to that seen for PC6-11 cells are seen with PC6-3 and PC6-15 cells in the absence and presence of ATA (not shown).

Discussion

Until recently, cell death was viewed as a passive process. The general view was that cells died because they ran out of molecules necessary for survival or that a series of molecular "errors" accumulated until basic cellular processes were compromised. It now appears that at least in a number of situations during development and possibly later in life, cells die because an endogenous "death program" is activated (Cohen and Duke, 1984; Ellis and Horvitz, 1986; Martin et al., 1988; Oppenheim et al., 1990; Schwartz et al., 1990). In parallel with this view of cells having a death program is the emerging view that one important function of growth factors may be to inhibit the activation of endogenous cell death genes (Duke and Cohen, 1986; Martin et al., 1988; McConkey et al., 1990; Oppenheim et al., 1990; Williams et al., 1990). A fundamental knowledge of genes that control cell death is important for understanding developmental events and could also provide potentially important information about pathophysiologies associated with the normal aging process and diseases.

An understanding of the mechanisms responsible for neuronal cell death require model systems for identifying molecules and pathways in the process. Useful properties of a model system
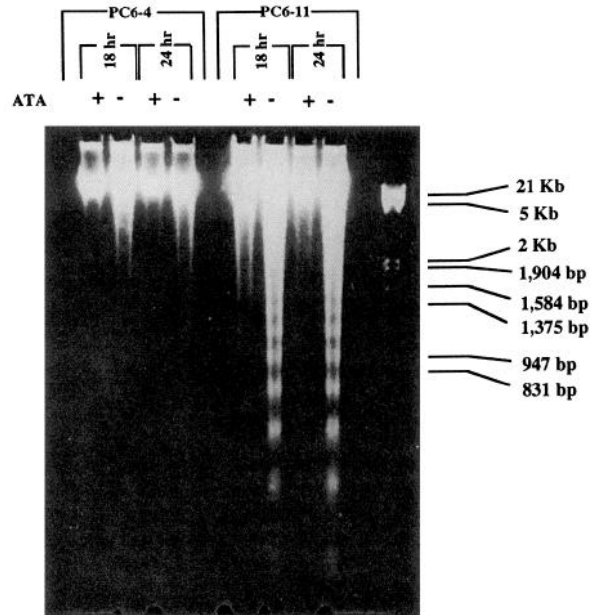
would include the following: (1) a homogeneous neuronal population, (2) cells available in large quantities, (3) death occurring with a reproducible time course either as part of a developmental program or following trophic factor removal, and (4) events identified *in vitro* accurately reflecting events *in vivo*. Our starting hypothesis was that if a neuronal cell line could be made to be dependent on a trophic factor such as NGF for survival, then it may serve as a good model system for identifying cellular and molecular events in cell death. The PC12 cell system described

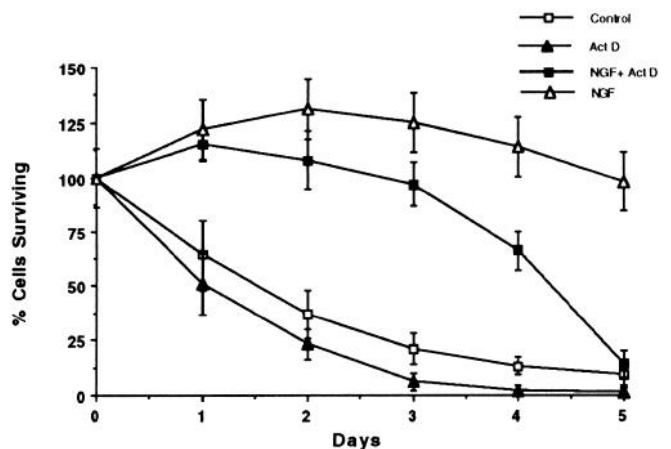*Figure 13.* Time course of survival of PC6-3 cells in the absence of agents (*open squares*) or in the presence of actinomycin D (*solid triangles*), or NGF (*open triangles*), or actinomycin D + NGF (*solid squares*) in serum-free medium lacking growth factors. Data represent means (±SD) of three different experiments performed in duplicate. Cell survival was determined using MTT and is expressed as a percentage of cells plated.
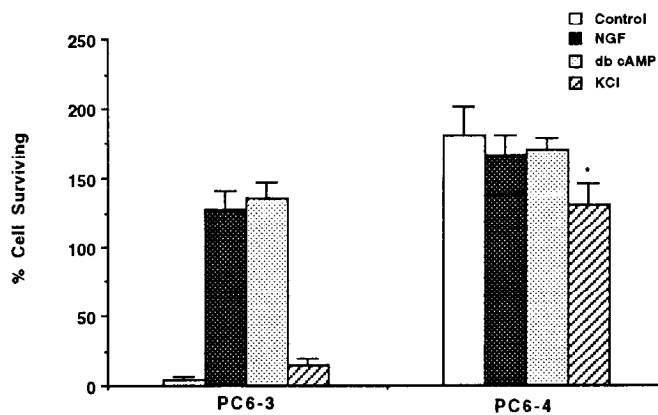
Figure 14. Survival of cells in the presence of NGF, dbcAMP, or KCl. Cell counts were made after 5 d in serum-free medium and represent means (±SD) of three different experiments performed in duplicate. Data are expressed as a percentage of cells plated. Survival of PC6-4 cells was significantly decreased in the presence of KCl compared to the other three groups (*, $p < 0.05$). Data similar to that obtained with PC6-3 were obtained with PC6-11 and PC6-15 cells.

in the present study has the advantages that it is more easily manipulated than in vivo systems and consists of a homogeneous population of cells available in large quantities. However, by definition cell lines are immortal, which raises an important issue concerning the ability of a cell line to reflect accurately the properties of neurons in vitro or in vivo. Although there is no way a priori to know if the PC12 system described here accurately reflects events in vivo, the data obtained with this system are similar to data obtained with sympathetic neurons in vitro (Martin et al., 1988) and with chick spinal motoneurons in vivo (Oppenheim et al., 1990). We have initiated studies to characterize genes upregulated in PC6-3 cells committed to die following removal of NGF. Information on whether these genes are also upregulated in primary cultures of neurons deprived of trophic support or in developing populations of neurons undergoing cell death will be important for determining whether or not PC6-3 cells are a useful model system for studying differentiated properties of neurons. A similar model system consisting of PC12 cells that undergo transcription-dependent cell death has been recently described by Koike (1992).

Experiments in the present study demonstrate that PC12 cells can be cultured under conditions that result in their becoming dependent on NGF for survival and following removal of NGF, cells undergo transcription-dependent cell death. The two factors that appeared to be most critical for converting PC12 cells into an NGF-dependent differentiated state were selecting an appropriate subline and then growing cells initially in a typical PC12 medium (RPMI containing 10% horse and 5% fetal calf serum) followed by subculturing cells and growing them in a medium more appropriate for primary cultures of neurons (DMEM/F12 containing 5% or 10% fetal calf serum or N2 serum-free components). As might be expected, there is no single point in time when the entire population of PC12 cells become dependent on NGF, but rather this occurs over several days. If cultures are grown in NGF for 5 d and then NGF removed, neurites degenerate but few if any cells die; however, starting around days 6–7 of NGF treatment, a small percentage of cells die when NGF is removed. The percentage of cells that die continues to increase over the next several days until by days 12–16, approximately 90% of the cells die following removal of NGF.

The sequence of events that occur following removal of NGF from PC12 cells are very similar to those occurring in sympathetic neurons following removal of NGF (Martin et al., 1988); however, events occur over a shorter time course in PC12 cells compared to sympathetic neurons. The progression of morphological events after removal of NGF from PC12 cells includes the following: (1) up until 10 hr after removal of NGF, essentially all cells can be rescued if NGF or actinomycin D is added to the culture; (2) by 12–14 hr after removal of NGF, 50% of the cells are committed to die during the next 12 hr; (3) initial morphological signs of cell death at the light microscopic level are apparent 14–18 hr after removal of NGF and involve thinning and "beading" of some neurites; (4) extensive fragmentation of neurites followed by active plasma membrane "blebbing" and degeneration of cell bodies occur between 20 and 36 hr.

An unexpected property of our system is that at approximately the same time cells become dependent on NGF for survival (days 6–10), spontaneous background death of cells (1–3% in PC6-3 cells and up to 10% in PC2 cells) becomes apparent even in the continual presence of NGF. The nature of the background death in our cultures is unknown, and because it could complicate future biochemical and molecular studies, particular efforts were directed at establishing conditions to minimize background cell death. Once biochemical or molecular markers are available for transcription-dependent cell death it will be interesting to determine if the spontaneous background death is due to a "leaky" death program that is not totally inhibited by NGF, or if it represents a different type of cell death. Addition of ATA does not block background cell death (Pittman, Wang, DiBenedetto, and Mills, unpublished observations); therefore, it does not appear to be the transcription-independent cell death that occurs in PC12 cells grown in serum-free medium in the absence of growth factors.

The PC12 system described in the present study in which cells undergo programmed cell death is different from that established by Greene's lab in which naive PC12 cells undergo transcription-independent cell death when shifted into serum-free medium in the absence of growth factors (Batistatou and Greene, 1991; Ruckenstein et al., 1991). A prominent feature of transcription-independent cell death in PC12 cells is activation of an endonuclease that results in laddering of DNA into 200 base pair nucleosomal fragments (Fig. 12; see also Batistatou and Greene, 1991). In agreement with previous studies (Batistatou and Greene, 1991; Ruckenstein et al., 1991), the endonuclease inhibitor ATA blocks transcription-independent cell death of our PC12 cells (Fig. 10). DNA laddering is present in a variety of cell types undergoing apoptotic cell death (for review, see Raff, 1992), and is postulated to be the cause of death in these cells (McConkey et al., 1989). Data in the present study are consistent with an endonuclease playing an important role in transcription-independent cell death of PC12 cells, but not in programmed (transcription-dependent) cell death. The only observation in our study inconsistent with those of Batistatou and Greene (1991) is that ATA does not have an appreciable effect on long-term survival of primary cultures of sympathetic neurons (Pittman, Wang, DiBenedetto, and Mills, unpublished observations), or differentiated PC12 cells (cells that undergo transcription-dependent death; Fig. 5). Under our tissue culture conditions (which are different from those used by Batistatou

and Greene, 1991), ATA increases survival of both sympathetic neurons and differentiated PC12 cells 10–20% at early times following removal of NGF (14–30 hr); however, it has no effect on survival of cells after 36 hr. Because ATA affects a large number of cellular enzymes and systems (Bina-Stein and Tritton, 1976), and fragmentation of DNA would unlikely contribute to cell death of postmitotic neurons at these early times following loss of trophic support (see discussion below), it is difficult to attribute these modest effects on cell survival to alterations of a specific set of cellular processes.

Besides differences in the role of endonucleases in cell death, several other major differences exist between transcription-independent and -dependent cell death of PC12 cells. These include differences in the ability of KCl, actinomycin D, and cycloheximide to block cell death, and the death of PC6-4 cells in the transcription-dependent paradigm following removal of NGF, but their survival in the transcription-independent paradigm following culturing in serum-free medium in the absence of growth factors. Characterizing PC6-4 cells as well as variants that do not die in serum-free medium should be useful for identifying cellular mechanisms that block transcription-independent cell death. Understanding these mechanisms may help identify cellular events involved in transcription-independent cell death.

The molecules and/or events responsible for killing a cell are unknown, but it seems reasonable that degrading DNA into nucleosomal fragments would be lethal for an actively proliferating cell. However, for a postmitotic cell such as a neuron that can live for several days in the absence of RNA synthesis (Figs. 5, 13; see also Martin et al., 1988), fragmentation of its DNA may not be acutely lethal. Therefore, other mechanisms are likely to be primarily responsible for programmed cell death of neurons (such as aberrant channels or enzymes, or loss of mitochondrial function), while endonuclease activation and DNA fragmentation may act as a backup mechanism to ensure that the neuron eventually dies if the primary mechanisms fail. Therefore, DNA fragmentation may be a common feature of apoptotic cell death and, depending on whether a cell is actively proliferating or postmitotic, may be a primary cause of death or a backup system to ensure that death eventually occurs.

Although the primary goal of the present study was to establish a model for characterizing cellular and molecular events in programmed cell death, the system described here should also be useful for identifying events involved in the transition of cells from a proliferative neuroblast state to a differentiated postmitotic neuronal phenotype. A large number of genes and proteins have been identified that are present in highly differentiated cells; however, little is known about cellular changes involved in converting cells from a proliferative to a postmitotic state. Downregulation of cdc2 and cyclin A have been correlated with the loss of proliferative capacity of neuronal precursors (Hayes et al., 1991), and increased DNA methylation and subsequent inactivation of genes is a property of many differentiated cells (Cedar, 1988; Bird, 1992). Having a homogeneous population of cells such as PC6-3 cells that is converted from an immortalized proliferative state to a differentiated nonproliferative state should be useful for identifying and characterizing events involved in this transition.

In summary, the availability of a large amount of tissue and a homogeneous population of cells should make PC6-3 cells a good model system for characterizing cellular, biochemical, and molecular events in programmed neuronal cell death. In addi-

tion, these cells should be useful for investigating molecular events involved in the transition of cells from a proliferative to a postmitotic state. Because these cells are derived from a clonal line, further characterization is needed before it can be determined if PC6-3 cells will be a useful model system for investigating other properties of primary cultures of neurons and/or neurons in vivo.

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